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A new species of *Calonectria* causing leaf disease of water lily in China

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Abstract — A species of *Calonectria*, isolated from a leaf spot of water lily (*Nymphaea tetragona*) in Guiyang, Guizhou Province, China was shown to be pathogenic by applying Koch's postulates. Identification based on morphological characters and a comparison of sequences from beta-tubulin and translation elongation factor 1 alpha (tef1) genes supported its status as a new species. *Calonectria nymphaeae* sp. nov. is introduced and compared with similar taxa.

Key words — ITS sequences, taxonomy, hyphomycetes

Introduction

During a survey of hyphomycetes in Guizhou Province, China, various taxa were isolated from different plant hosts. Two strains isolated from a leaf spot of water lily had branched conidiophores and a stipe extension terminating in characteristic vesicles, producing cylindrical, 1–multi-septate conidia; these morphological characteristics are typical of *Cylindrocladium* Morgan (anamorphic *Calonectria* De Not.) (Crous & Wingfield 1994, Crous 2002). In culture the strain also produced a *Calonectria* teleomorph. Recent studies have largely improved our understanding of the phylogeny and systematics among *Calonectria* and allied genera (Lombard et al. 2010a,b,c, Doveri et al. 2010). Lombard et al. (2010c) provided dichotomous and synoptic keys to all *Calonectria* species currently recognized.

The *Calonectria* species isolated from *Nymphaea tetragona* is described as a new species, *C. nymphaeae*. It is illustrated with light micrographs and its uniqueness is confirmed in a phylogram generated from beta-tubulin and translation elongation factor 1 alpha (TEF1) sequence data, which are the regions that provide most valuable insights into relationship between all *Calonectria* species (Schoch et al. 2000, 2001, Crous 2002, Henricot & Culham 2002). Koch's postulates also confirmed that this new taxon was the causal agent of leaf disease of water lily.

Materials & methods

Morphological and cultural studies

Diseased leaves of *Nymphaea tetragona* growing in a pond in Huaxi, Guiyang city, Guizhou Province, China were collected in clean plastic bags and returned to the laboratory. Small tissue pieces (each approximately 5 × 5 mm) were cut from the boundary between healthy and infected parts of leaves, which were previously surface disinfected with 0.5% NaOCl. The pieces were plated onto malt extract agar (2% w/v; MEA; Biolab, Midrand, South Africa) and incubated at 25°C for 7 days under continuous near-UV light. Mycelia grew from the infected samples and were transferred to fresh MEA plates. Cultural characteristics and morphology were determined on MEA, and carnation leaf agar (CLA) [1% water agar (10 g/L) with autoclaved carnation leaves placed onto the medium] (Gams *et al.* 1998) and incubated for 7 days at 25°C under continuous near-UV light, to promote sporulation. A water solution of 60% (v/v) lactic acid without a color dye was used as the mounting medium. Slides were examined under oil immersion with a UB200i microscope (Chongqing UOP Photoelectric Technology, China) at 1000× magnification. Dried cultures of this species were deposited in the Plant Pathology Herbarium of Guizhou University (HGUP). Living cultures were deposited in HGUP and Centraalbureau voor Schimmelcultures (CBS).

DNA sequencing and alignment

DNA was extracted from mycelium using CTAB method. Three loci including fragments of the ITS, β-tubulin (BT), and translation elongation factor 1 alpha (TEF1) gene regions were sequenced. Primers used to sequence these regions were ITS1 and ITS4 (White et al. 1990) for ITS region, T1 (O'Donnell & Cigelnik 1997) and CYLTUB1R (Crous et al. 2004) for the BT region, and EF1-728F (Carbone & Kohn 1999) and EF2 (O'Donnell et al. 1998) for the TEF1 region. Reaction mixtures contained 5 µL of 10 × ThermoPol reaction buffer [200 mM Tris-HCl, pH 8.3, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$ and 1% Triton X-100], 5 µL of 10 mM $MgSO_4$, 20 ng template genomic DNA, 4 pM of each primer, 4 µL of 2.5 mM dNTPs, 2.5 U of AmpliTaq polymerase, and total volume was adjusted to 50 µL with deionized water. The PCR amplified DNA fragments were fractionated in 1% agarose gels in 0.5 × TBE buffer, and DNA was visualized by ethidium bromide staining and UV illumination. Sequencing was performed with an ABI PRISM 3730 DNA autosequencer using either dRhodamine terminator or Big Dye Terminator chemistry (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California). The DNA sequences of isolates HGUP100003 and

HGUP100004 in ITS, BT and TEF1 regions generated in this study were submitted to GenBank (JF499828, JN984864, JN984866, JN984865, JX546573 and JX546579). The ITS sequences comparison with published sequences was performed using a BLAST search.

Phylogenetic analyses

The combined BT and TEF1 DNA sequence of HGUP100003 was primarily aligned with the ClustalX (Thompson et al. 1997). Alignments files are available in TreeBASE (www.treebase.org/treebase-web/home.html) with study ID 12047. We built up the phylogenetic tree based on combined BT and TEF1 gene regions using the MP (Maximum Parsimony) by PAUP* 4.0b10 (Swofford 2002) and Bayesian methods by MrBayes v3.1.1 (Ronquist & Huelsenbeck 2003). A partition homogeneity test (Farris et al. 1994) was applied to evaluate the feasibility of combining the datasets. In the MP analyses, trees were inferred using a heuristic search option with tree bisection reconnection (TBR) branch swapping and 1000 random sequence additions replicates, maxtrees were 100, branches of zero length were collapsed and all parsimonious trees were saved. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analyses (Hillis & Bull 1993) were based on 1000 replications.

The model of evolution in Bayesian analysis was TPM2uf+G estimated by jModelTest 0.0.1 (Posada 2008). A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v3.1.1 for the BT and TEF1 sequence datasets. Two independent runs of four MCMC chains were run simultaneously from random trees for 1,010,000 generations and sampled every 100 generations for the combined analysis of the gene partitions. Both runs converged on the same likelihood score and tree topology, and the first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from the remaining 7600 trees.

Pathogenicity test

Pathogenicity of this fungus was determined by inoculating healthy leaves of *N. tetragona* with 5 mm diameter mycelial plugs, cut from the margins of 10-day-old actively growing cultures; the control was treatment with sterile agar plugs. Both inoculated and control plants were kept in a moist chamber at 25°C for 4 days, and observed for symptom development. Infected leaves were collected and the fungus was reisolated in MEA medium and compared with the original isolate.

Results

Phylogenetic analysis

Our initial BLAST searches identified DNA sequences of isolates HGUP100003 and HGUP100004 to be most closely related to those of *Calonectria eucalypti*. The alignment sequences indicated the ITS sequence of this fungus was 99.6% identical (only two base pairs difference) to four sequences (GQ280631, GQ280632, GQ280633, GQ280634) of *C. eucalypti* with 499-bp characters. Sequence GQ280633 originated from the *C. eucalypti* ex-

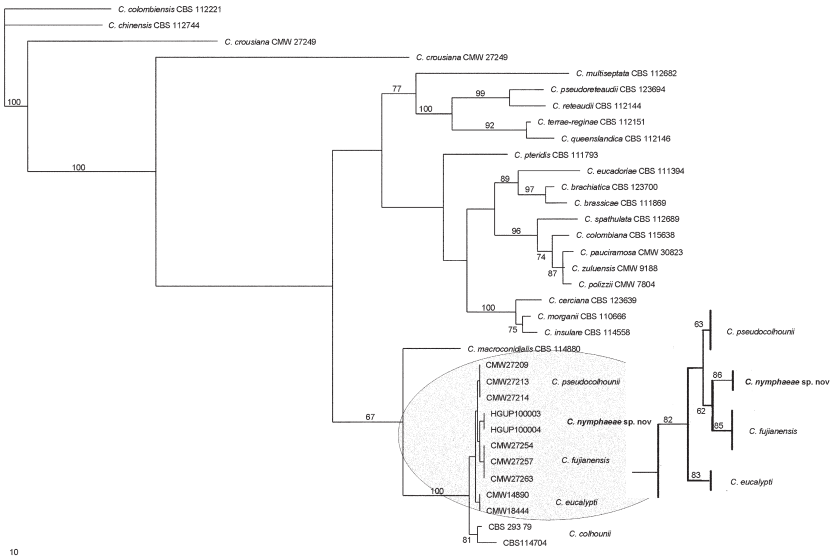

Fig. 1 One parsimonious tree based on combined sequences of BT (beta-tubulin) and *tef1* (translation elongation factor 1 alpha) for *Calonectria nymphaeae* and 24 other *Calonectria* species downloaded from GenBank. *C. colombiensis* (CBS 112221) and *C. chinensis* (CBS 112744) serve as outgroups. The detailed phylogenetic relationships between *C. nymphaeae*, *C. fujianensis*, *C. eucalypti*, and *C. pseudocolhounii* are shown in the amplified part of the bottom right corner. Bootstrap values ≥ 50% are shown above or below branches. Thickened branches indicate Bayesian PP ≥ 95 %.

type strain (CMW18444); therefore we originally identified our two isolates as *C. eucalypti*.

However, *C. eucalypti* is a species complex and our *Calonectria* isolates were revealed as a cryptic species following comparisons of sequences of BT and TEF1 gene regions. Partition homogeneity tests for combinations of the two gene regions used, yielded a P-value of 0.001. Based on the tree topologies and a P-value of 0.001 (Cunningham 1997, Dettman et al. 2003), the two gene regions were combined. The aligned sequence data matrix contained 27 taxa, including 2 outgroups and 1024 characters. Among them, BT has 515 characters, and TEF1 has 509 characters. Of these, 294 were parsimony informative. Ten most parsimonious trees were obtained, one to represent the topology of the strict consensus tree selected for presentation (Fig. 1). The tree was described as follows: Tree Length (TL) = 833, CI = 0.6423, RI = 0.8012, HI = 0.3577, and RC = 0.5146. In this parsimonious tree, 25 species of *Calonectria* resided in a strongly supported large monophyletic clade with a bootstrap value of 100%.

Among them, two isolates of *C. nymphaeae* clustered together with a 86% supported value. *Calonectria nymphaeae*, *C. pseudocolhounii*, *C. eucalypti*, *C. colhounii*, and *C. fujianensis* are related species supported by a high bootstrap value (100%). However, only *C. fujianensis* showed a closer relationship with *C. nymphaeae* with a moderate bootstrap value (62%), and *C. nymphaeae* and *C. eucalypti* are distant.

The topology in Bayesian analysis was nearly identical to that of the MP analyses. The Bayesian trees are therefore not shown, but the statistically supported clades (posterior probabilities ≥ 0.95) are marked with a thickened line in the parsimony tree (Fig. 1).

Taxonomy

Calonectria nymphaeae Yong Wang bis, S.Y. Qin, P. Tan & K.D. Hyde, **sp. nov.**

MycoBank MB 800112 Fig. 2

Differs from *Calonectria pseudocolhounii* and *C. fujianensis* by its slightly larger macroconidia and its longer, narrower ascospores.

Type: China, Guizhou Province, Guiyang, Huaxi, Huaxi Garden, in a pond, from the diseased leaves of *Nymphaea tetragona* Georgi (*Nymphaeaceae*), dried sporulating MEA medium cultures, July 2010, S.Y. Qin (**Holotype**, HGUPd100003; **isotype**, CBS; ex-type living cultures—HGUP100003, CBS 131802; GenBank—JF499828, JN984864, JX546573).

Etymology: in reference to the host from which the fungus was isolated.

Ascomata solitary or in groups, orange to red, becoming red-brown with age, perithecial; in section, apex and body yellow to orange, base red-brown, subglobose to ovoid, 360–450 µm high, 320–380 µm diam. Peridium rough, consisting of 2 thick-walled layers; outer layer of textura globulosa, 30–60 µm wide; becoming more compressed towards inner layer of textura angularis, 13–16 µm wide; becoming thin-walled and hyaline towards the center, outer cells 28–35 × 10–18 µm; inner cells 10–13 × 5–7 µm, ascomata base up to 90–100 µm wide, consisting of dark red, angular cells, merging with an erumpent stroma, cells of the outer wall layer continuing into the pseudoparenchymatous cells of the erumpent stroma. Asci 4-spored, clavate, 100–145 × 12–16 µm, tapering to a long thin stalk. Ascospores aggregated in the upper third of the ascus, hyaline, guttulate, fusoid with rounded ends, straight to slightly curved, 2–3-septate, not or slightly constricted at the septa, 51–71 × 5.5–6.5 µm (av. = 66 × 6.0 µm). Cultures homothallic. Conidiophores with a stipe bearing a penicillate suite of fertile branches, stipe extensions, and terminal vesicles. Stipe septate, hyaline, smooth, 250–300 × 3–4.5 µm; stipe extensions septate, straight to flexuous, 120–240 µm long, 3–5 µm wide at the apical septum, terminating in a clavate vesicle, 3–5 µm diam. Conidiogenous apparatus 50–100 µm long, and 30–70 µm wide; primary branches aseptate or 1-septate, 18–28 × 4–5

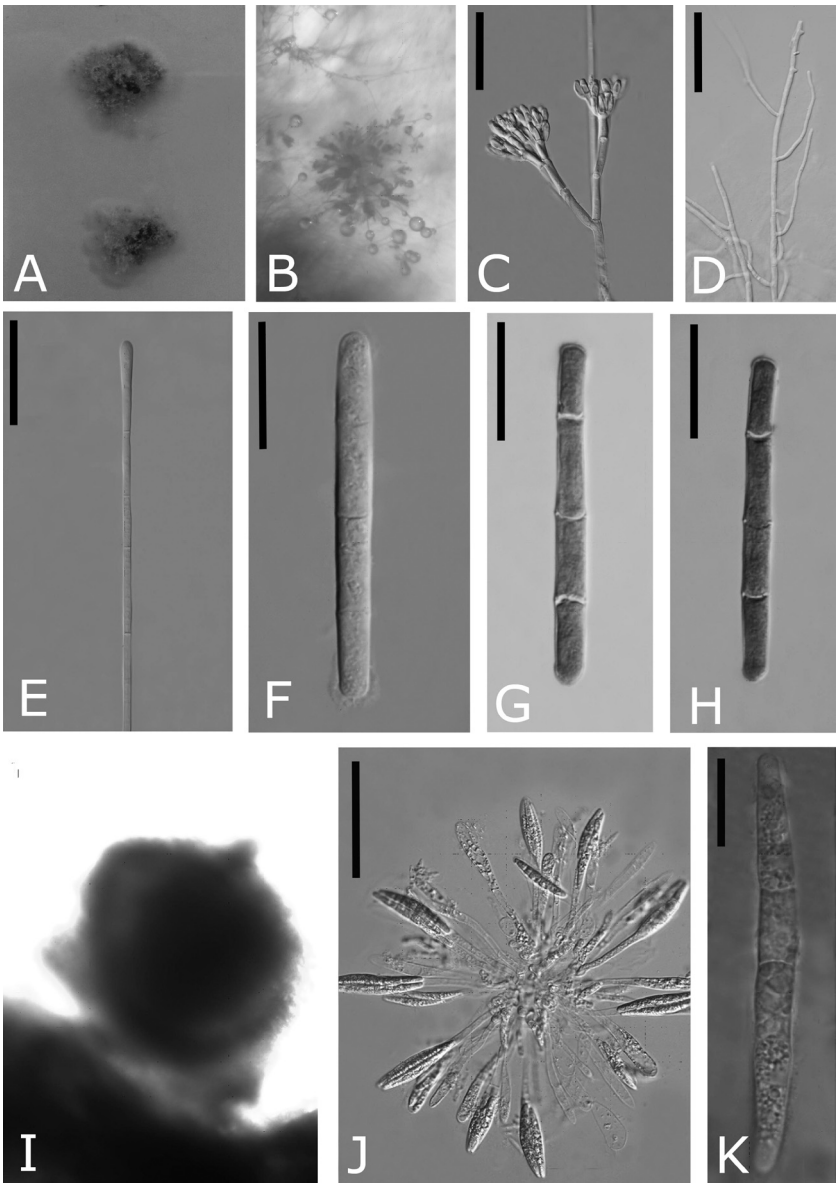

Fig. 2 *Calonectria nymphaeae*: A–B. cultures on MEA; C. macroconidiophore; D. fertile branches; E. clavate vesicle; F. macroconidium; G–H. macroconidia stained by cotton blue; I. perithecium; J. asci; K. ascospore. Scale bars: C–E = 50 μm; F–H = 20 μm; J–I = 100 μm; K = 15 μm.

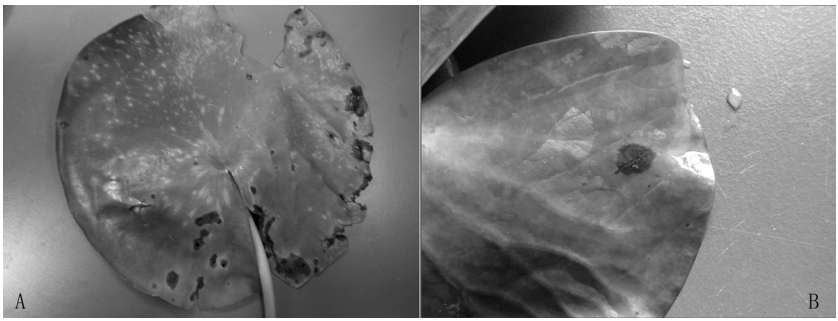

Fig. 3 A: Naturally occurring symptoms on *Nymphaea tetragona* caused by *Calonectria nymphaeae*; B: Symptoms on *N. tetragona* leaves by inoculation.

μm; secondary branches aseptate, 9–15 × 3–4 μm; tertiary branches aseptate, 6–12 × 2.5–3.5 μm, each terminal branch producing 2–4 phialides; phialides doliiform to reniform, hyaline, aseptate, 9–12 × 2.5–3 μm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, 55–63 × 5.3–6.3 μm (av. = 61 × 5.9 μm), 3–4-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime.

Additional specimen examined: **China**, **Guizhou Province**, Guiyang, Huaxi, in a pond, from diseased leaves of *Nymphaea tetragona*, August 2011, Y. Wang, (HGUP100004; GenBank—JF984866, JN984865, JX546579).

Comments: *Calonectria nymphaeae* is similar to species in the *C. colhounii* complex (Chen et al. 2011) that all have yellow ascomata, (1–)3-septate ascospores, and clavate vesicles in the anamorph state. *Calonectria nymphaeae* is morphologically most similar to *C. pseudocolhounii* S.F. Chen et al. and *C. fujianensis* S.F. Chen et al. However, the macroconidia of *C. nymphaeae* are slightly larger than those of *C. pseudocolhounii* (av. 60 × 4.5 μm) and *C. fujianensis* (av. 52.5 × 4.5 μm). The ascospores of *C. nymphaeae* are longer but narrower than those of *C. pseudocolhounii* (av. 56 × 6.5 μm) and *C. fujianensis* (av. 55.5 × 6.8 μm). In addition, *C. pseudocolhounii* and *C. fujianensis* were isolated from terrestrial hosts (*Eucalyptus dunnii* and *E. grandis*), while *C. nymphaeae* was isolated from a hydrophyte.

Pathogenicity test

Following mycelial inoculation, *N. tetragona* leaves exhibited dark brown to black necrotic spots (Fig. 3b) after 4 days, which were very similar to those of natural infection (Fig. 3a). *C. nymphaeae* was successfully reisolated from the artificially inoculated leaves of *N. tetragona*, thus establishing proof of pathogenicity. Controls remained healthy and the leaves did not yield any microorganisms at 4 days.

Discussion

The phylogenetic results indicate that ITS sequence data poorly resolves closely related species of *Calonectria*, while BT and TEF1 sequence data are more effective DNA markers for phylogenetic analyses. In our study, morphological comparison indicated that *C. nymphaeae* possessed some unique characters that differ from related species. The phylogenetic analyses based on BT and TEF1 regions confirmed that the two *C. nymphaeae* isolates clustered together as an independent branch with creditable bootstrap (86%) and posterior probabilities values (1.00) (FIG. 1). The clade including *C. nymphaeae*, *C. pseudocolhounii*, *C. eucalypti*, *C. colhounii*, and *C. fujianensis* was supported by high statistical values (BP 100%, PP 1.00), which is consistent with morphological comparison. By combining morphology and phylogeny we conclude that *C. nymphaeae* is a novel species. Koch's postulates showed the taxon to be the causal agent of the leaf spot disease of *N. tetragona*. According to Peerally (1991), *Cylindrocladium hawksworthii* Peerally (anamorphic *Calonectria hawksworthii* L. Lombard et al.) was the pathogen of a common foliage disease on *Nymphaea lotus* in Mauritius. However, *C. nymphaeae* produces conidia with 3–4 septa, but those of *Cylindrocladium hawksworthii* have only 1 septum. The phylogenetic analyses also proved these two pathogens were not the same species.

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